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Characterization and Sequence Analysis of a Small Cryptic Plasmid from *Lactobacillus curvatus* LTH683 and Its Use for Construction of New *Lactobacillus* Cloning Vectors

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Lactobacillus curvatus LTH683, a strain originally isolated from raw sausage, contains the single cryptic plasmid called pLC2. The sequence and genetic organization of the complete 2489-bp plasmid pLC2 was determined and used as the basis for construction of a series of vectors useful in *Lactobacillus* strains. The major parts of pLC2 nucleotide sequence could be aligned with other plasmids from gram-positive bacteria replicating by a rolling circle mechanism of replication (RCR). Direct evidence for a RCR mechanism was obtained by showing the accumulation of single-stranded plasmid intermediates in the presence of rifampicin. Three protein-coding sequences could be predicted and the corresponding proteins were detected after *in vitro* transcription/translation of pLC2 plasmid DNA. ORFs 1 and 3 showed minor homologies to plasmids of gram-positive bacteria. The replication protein coded by ORF2 and its corresponding target sequence, the plus origin, were similar to replication regions of other gram-positive bacteria plasmids like pLS1, pWV01, and pE194. Upstream of the *ori*+ site, in a noncoding region, which was nonessential for replication, strong homology to other *Lactobacillus* plasmids like pC30i1, pLP1, pLJ1, and pLAB1000 could be detected. A palindromic sequence predicted to be the minus origin of replication was localized there. Small vectors (3213 bp) suitable for cloning in lactobacilli were constructed based on a 1635-bp DNA fragment of pLC2, containing the region necessary for replication, marked with the chloramphenicol resistance gene and a multiple cloning site. © 1993 Academic Press, Inc.

In recent years there has been an upsurge in interest into the possibility of genetically manipulating lactic acid bacteria (McKay and Baldwin, 1990). Many species are now transformable by using the electroporation technique and thus become amenable to recombinant DNA technology. A strategy for the construction of cloning vectors is to use the origins of replication of small multicopy cryptic plasmids from lactobacilli and to incorporate selectable markers.

Very little is known about the replication mode of plasmids in lactobacilli. Characterizations of plasmids isolated from different *Lactobacillus* species (Bates and Gilbert, 1989; Bouia *et al.*, 1989; Cocconelli *et al.*, 1991; Josson *et al.*, 1990; Leer *et al.*, 1992; Skaugen, 1989) have revealed that they may belong to the group of single-stranded (ss) DNA plasmids that replicate via a RCR mechanism (Gruss and Ehrlich, 1989; Te Riele *et al.*, 1986). The nucleotide sequence data indicate that all *Lactobacillus* plasmids analyzed so far have a similar organization as ssDNA plasmids from other gram-positive bacteria. All plasmids encode a site-specific nuclease, the Rep protein, which produces a specific single-stranded nick at the plus origin that allows initiation of synthesis of the leading DNA strand. Depending on the similarity of plus origin sequences, the various plasmids have been classified into three groups (Gruss and Ehrlich, 1989). Several *Lactobacillus* plasmids contain palindromic structures which resemble the minus origin (MO) of replication in ssDNA plasmids. The MOs are involved in the conversion of ssDNA into dou-

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ble-stranded (ds) DNA. These nonessential regions have been distinguished into three classes (Gruss and Ehrlich, 1989) but additionally MOs of *Lactobacillus* plasmids have been described, which have no homology to MOs of other ssDNA plasmids (Leer *et al.*, 1992). Since inserts of foreign DNA might influence plasmid stability (Gruss and Ehrlich, 1988), efforts were made to isolate large lactobacillal plasmids replicating via a double-stranded theta-type mechanism (Jannière *et al.*, 1990) as it is described for the well-characterized plasmid pAMβ1 isolated from *Enterococcus faecalis* (Leblanc and Lee, 1984), but until now no report of such a plasmid has been published.

Lactobacillus curvatus is a commercially important microorganism in sausage fermentation and like many other lactobacilli this species harbors cryptic plasmids. The presence of plasmid DNA in *L. curvatus* strains has been reported (Vogel *et al.*, 1991) and plasmid transfer and segregation of transformed vector plasmids was investigated (Vogel *et al.*, 1992). The segregationally highly stable small cryptic plasmid pLC2 of *L. curvatus* LTH683 (formerly LC2) could serve as a minimal replicon in food-grade vectors for use in lactobacilli, especially for *L. curvatus*, a species with a plasmid cured and transformable strain (Gaier *et al.*, 1990). Recently a catalase gene of this species was cloned, which has the potential to be a food grade marker gene (Knauf *et al.*, 1992), but until now no vector with a replicon originating from this species was available. The analysis of plasmid pLC2, which was used to construct such cloning vectors, is also a contribution in the studies of the mode of replication of small plasmids from gram-positive bacteria, which are to proceed by a RCR mechanism.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains used are summarized in Table 1. *Escherichia coli* and *Bacillus subtilis* were grown at 37°C in Luria broth (Miller, 1972), *Lactococcus lactis* was propagated in M17 supplemented with 0.5% glucose (GM17) (Terzaghi and Sandine, 1975) and lactobacilli in MRS (De Man *et al.*, 1960) at 30°C. Ampicillin and chloramphenicol were added to concentrations of 100 and 10 μg/ml, respectively.

Plasmid pJK80 is a positive selection vector for insert DNA based on pUH84 (Henrich and Plapp, 1986), with a chloramphenicol resistance (Cm^R) gene as a marker in gram-positive bacteria, but which is not able to replicate in Gram positives, unless a functional replicon is inserted. The Cm^R gene was isolated as a 1035-bp *Hpa*II/*Sau*3A fragment from pGK12 (Kok *et al.*, 1984), treated with Klenow enzyme and ligated into the filled-in *Nde*I site of pUH84. After integration of the functional replicational region of pLC2, the hybrid plasmid, could be selected in lactobacilli with chloramphenicol. The cryptic plasmid pLC2 was isolated from *L. curvatus* LTH683.

Recombinant DNA Techniques

Restriction enzymes and other nucleic acid-modifying enzymes were used as recommended by the manufacturers. Isolation of plasmid DNA from *E. coli* was performed as described by Sambrook *et al.* (1989). Plasmid DNA isolation from *L. casei* or *L. curvatus* was performed as described elsewhere (Zink *et al.*, 1991).

Inhibition of RNA Polymerase by Rifampicin and Detection of Single-Stranded DNA in Cell Lysates of L. curvatus LTH683

For accumulation of ssDNA, cultures of *L. curvatus* were incubated with Rifampicin as described previously (Leenhouts *et al.*, 1991) and whole-cell lysates were prepared with slight modifications. The lysis buffer of 10 mM Tris–HCl, 1 m*M* EDTA, pH 8.0, was

TABLE 1

BACTERIAL STRAINS

Strain		(Formerly referred to as)	Reference
Lactobacillus casei	LK1	(WS97)	Zink *et al.* (1991)
Lactobacillus curvatus	LTH683	(Lc2)	Vogel *et al.* (1992)
Lactobacillus curvatus	LTH1432	(Lc2-c)	Vogel *et al.* (1992)
Lactococcus lactis ssp. *lactis*	MG1363		Gasson (1983)
Bacillus subtilis	6GM		Haima *et al.* (1990)
Escherichia coli K-12	MM294		Hanahan (1983)

supplemented with lysozyme (10 mg/ml) and mutanolysin (500 U/ml) and cells were incubated at 37°C for 15 min. Lysates were incubated with Sarkosyl for 15 min at 60°C. After phenol extractions, crude lysates were suspected to agarose gel electrophoresis for detection of ssDNA accumulation. Prior nuclease S1 treatment the DNA was precipitated and washed with 80% ethanol. After electrophoresis in a agarose slab, the DNA was transferred to a nylon membrane (Hybond, Amersham). The presence of ssDNA was determined by Southern hybridization with digoxigenin-dUTP labeled pLC2 DNA as probe. Labeling and detection of the DNA was carried out using the nonradioactive digoxigenin kit (Boehringer-Mannheim, Germany).

Cloning of the pLC2 Origin in pJK80

The unique *Sal*I site of the cryptic plasmid pLC2 was used to produce a linear molecule of 2.5 kb in size. Following the strategy described earlier (Zink *et al.*, 1991), we were able to clone this fragment with the functional origin into the *Sal*I site of pJK80, thus inactivating the lysis gene E. Hybrid plasmids were isolated from *E. coli* and introduced into *L. casei* LK1 by electroporation. The chloramphenicol resistance marker of plasmid pJK80, originating from pC194 (Horinouchi and Weisblum, 1982), served as a selectable marker in lactobacilli. Plasmid pCU1882 could then be isolated from chloramphenicol resistant transformants.

DNA Sequence Analysis

For nucleotide sequencing of inserts in pJK80, a pair of universal sequencing primers, adjacent to the multiple cloning site, was synthesized. Synthetic oligonucleotide primers, deduced from the investigated sequence, were synthesized and allowed direct sequencing of double-stranded pLC2 plasmid DNA. This proved that the whole plasmid pLC2 is represented by the *Sal*I restriction fragment cloned in vector pJK80.

The DNA sequence of each strand was determined using the T7 DNA polymerase sequencing kit (Pharmacia, Uppsala, Sweden), which is based on the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) in the presence of [α-^{35}S]dATP (Amersham). For computer-assisted sequence analysis the Microgenie (Beckman, Palo Alto, CA), PC-Gene (IntelliGenetics, East El Camino Real, CA), and HUSAR (GENIUSnet, Heidelberg) software were used.

Sequencing primers were synthesized using Applied Biosystems Model 392 DNA synthesizer and reagents.

Transformations

L. casei, *L. curvatus*, Lactococcus *lactis*, and *E. coli* cells were transformed by electroporation using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, CA) as described before (Zink *et al.*, 1991; Gaier *et al.*, 1990; Vos *et al.*, 1989; Dower *et al.*, 1988). Preparation of competent *B. subtilis* cells and transformation with plasmid DNA were per-

formed according to the Groningen Method (Bron, 1990).

DNA-Directed in Vitro Transcription/ Translation and Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE) Electrophoresis

In vitro transcription and translation was performed with plasmid DNA purified by CsCl density gradient centrifugation, using a cell-free coupled transcription-translation system derived from *E. coli* (Amersham). Synthesized proteins labeled with L-[^{35}S]-methionine were separated by SDS–PAGE on vertical slab gels using the Laemmli (1970) buffer system and detected by autoradiography.

Determination of Plasmid Stability

Strains LK1 and LTH1432 transformed with plasmids pCU1882, pJK355, and pJK356 were incubated without selective pressure. After reaching the stationary phase the culture was diluted by 1:16384 for a further cycle, thus reaching approximately 14 generations per cycle. Loss of plasmid was measured by counting the survivals plated on agar plates with and without the selective antibiotic chloramphenicol. For determination of plasmid content and plasmid integrity, DNA preparations of chloramphenicol-resistant colonies were examined by electrophoresis.

Host Range of pLC2 Replicon

For determination of host range, the strains listed in Table 1 were transformed with plasmids pJK351, pJK355, and pJK356, having only the unique origin of pLC2, and selected for resistance against chloramphenicol (10 μg/ml; *L. lactis* MG1363 was selected with 20 μg/ml). In *E. coli* resistance had to be induced in subinhibitory levels (500 ng/ml) (Horinouchi and Weisblum, 1982). Transformed colonies were grown to stationary phase in liquid culture and subjected to plasmid isolation.

RESULTS AND DISCUSSION

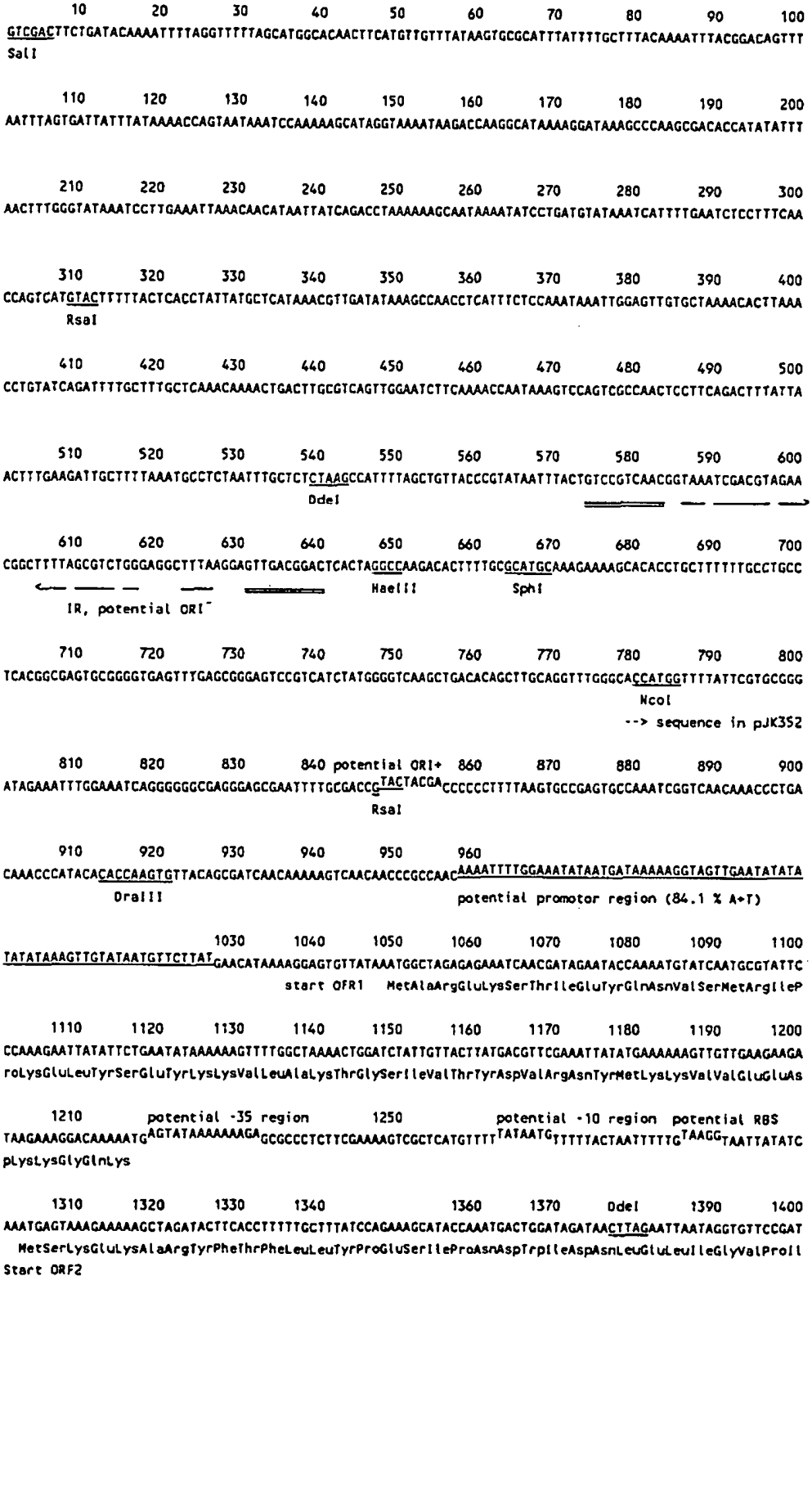
Characterization of the Cryptic Plasmid pLC2

General features of the nucleotide sequence. The G+C content of the cryptic *Lactobacillus* plasmid pLC2 was 33.6%, which is low in respect to the 43% reported for the chromosome (Dellaglio *et al.*, 1973). Computer-assisted analysis revealed the presence of three open reading frames, potentially capable of encoding polypeptides with molecular sizes of 6633, 25688, and 17210 D (Fig. 1).

The establishment of the pLC2 sequence and the determination of the essential replicational region enabled the construction of pJK355 and pJK356 to be used as small cloning vectors with multiple cloning sites.

Nucleotide sequence homologies. The complete nucleotide sequence of pLC2 was searched against the EMBL database, with the result that the major part of the plasmid has a significant degree of homology in numerous plasmids from gram-positive bacteria which are all replicating by a RCR mechanism. The degree of similarity on the nucleotide level is summarized in Fig. 2, which demonstrates that almost the entire pLC2 plasmid has homology to plasmids which have themselves origins of distinct groups. Long stretches of homology, discussed below in more detail, were found for the regions containing the proposed plus and minus origins and for the ORF1 and ORF2 stretches. Only few homologous sequences were found for the nonessential and deletable regions of pLC2.

The pLC2 plus ori+ site. By comparing the sequence with three types of plus origin sequences pT181, pC194, or pE194 (Gruss and Ehrlich, 1989), a region homologous to the plus *ori* sites of the pE194 group plasmids was identified (Fig. 3A). The location of the *ori* site upstream and not within the proposed protein coding regions in pCU1882 (Fig. 4) was confirmed by deleting the 924-bp *Pst*I–


10 20 30 40 50 60 70 80 90 100
GTCGACTTCTGATACAAAATTTTAGGTTTTTAGCATGGCACAACTTCATGTTGTTTATAAGTGCGCATTTATTTTGCTTTACAAAATTTACGGACAGTTT
SalI
110 120 130 140 150 160 170 180 190 200
AATTTAGTGATTATTTATAAAACCAGTAATAAATCCAAAAAGCATAGGTAAAATAAGACCAAGGCATAAAAGGATAAAGCCCAAGCGACACCATATATTT
210 220 230 240 250 260 270 280 290 300
AACTTTGGGTATAAATCCTTGAAATTAAACAACATAATTATCAGACCTAAAAAAGCAATAAAATATCCTGATGTATAAATCATTTTGAATCTCCTTTCAA
310 320 330 340 350 360 370 380 390 400
CCAGTCATGTACTTTTTACTCACCTATTATGCTCATAAACGTTGATATAAAGCCAACCTCATTTCTCCAAATAAATTGGAGTTGTGCTAAAACACTTAAA
RsaI
410 420 430 440 450 460 470 480 490 500
CCTGTATCAGATTTTGCTTTGCTCAAACAAAACTGACTTGCGTCAGTTGGAATCTTCAAAACCAATAAAGTCCAGTCGCCAACTCCTTCAGACTTTATTA
510 520 530 540 550 560 570 580 590 600
ACTTTGAAGATTGCTTTTAAATGCCTCTAATTTGCTCTCTAAGCCATTTTAGCTGTTACCCGTATAATTTACTGTCCGTCAACGGTAAATCGACGTAGAA
DdeI
610 620 630 640 650 660 670 680 690 700
CGGCTTTTAGCGTCTGGGAGGCTTTAAGGAGTTGACGGACTCACTAGGCCAAGACACTTTTGCGCATGCAAAGAAAAGCACACCTGCTTTTTTGCCTGCC
HaeIII
SphI
IR, potential ORI⁻
710 720 730 740 750 760 770 780 790 800
TCACGGCGAGTGCGGGGTGAGTTTGAGCGGGAGTCCGTCATCTATGGGGTCAAGCTGACACAGCTTGCAGGTTTGGGCACCATGGTTTTATTCGTGCGGG
NcoI
--> sequence in pJK352
810 820 830 840 potential ORI+ 860 870 880 890 900
ATAGAAATTTGGAAATCAGGGGGGCGAGGGAGCGAATTTTGCGACCGTACTACGACCCCCCTTTAAGTGCCGAGTGCCAAATCGGTCAACAAACCCTGA
RsaI
910 920 930 940 950 960
CAAACCCATACACACCAAGTGTTACAGCGATCAACAAAAAGTCAACAACCCGCCAACAAAATTTTGGAAATATAATGATAAAAAGGTAGTTGAATATATA
DraIII
potential promotor region (84.1 % A+T)
1030 1040 1050 1060 1070 1080 1090 1100
TATATAAAGTTGTATAATGTTCTTATGAACATAAAAGGAGTGTTATAAATGGCTAGAGAGAAATCAACGATAGAATACCAAAATGTATCAATGCGTATTC
start ORF1 MetAlaArgGluLysSerThrIleGluTyrGlnAsnValSerMetArgIleP
1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
CCAAAGAATTATATTCTGAATATAAAAAAGTTTTGGCTAAAACTGGATCTATTGTTACTTATGACGTTCGAAATTATATGAAAAAAGTTGTTGAAGAAGA
roLysGluLeuTyrSerGluTyrLysLysValLeuAlaLysThrGlySerIleValThrTyrAspValArgAsnTyrMetLysLysValValGluGluAs
1210 potential -35 region 1250 potential -10 region potential RBS
TAAGAAAGGACAAAAATGAGTATAAAAAAAGAGCGCCCTCTTCGAAAAGTCGCTCATGTTTTTATAATGTTTTTACTAATTTTTGTAAGGTAATTATATC
pLysLysGlyGlnLys
1310 1320 1330 1340 1360 1370 DdeI 1390 1400
AAATGAGTAAAGAAAAAGCTAGATACTTCACCTTTTTGCTTTATCCAGAAAGCATACCAAATGACTGGATAGATAACTTAGAATTAATAGGTGTTCCGAT
MetSerLysGluLysAlaArgTyrPheThrPheLeuLeuTyrProGluSerIleProAsnAspTrpIleAspAsnLeuGluLeuIleGlyValProIl
Start ORF2

```
     1410      1420      1430      1440                1460      1470      1480      1490      1500
TGCTATTAGTCCTCTGCATGATAAGGATTTAAGTGATGTTGAGGGACAAAAATATAAAAAAGAACATTATCATGTAATCTATGTTTCAAAAAATCCTGTA
eAlaIleSerProLeuHisAspLysAspLeuSerAspValGluGlyGlnLysTyrLysLysGluHisTyrHisValIleTyrValSerLysAsnProVal

     1510      1520      1530      1540      1550      1560      1570  RsaI          1590      1600
ACTGCTGAAAGTGTGCGTTTGAAAGTTAAGCGCTCTTTGGGAGATAAAAGTGTTGCTATGGTTCAGATTGTAAGTACGAGTATGGAAAATATGTATTTGT
ThrAlaGluSerValArgLeuLysValLysArgSerLeuGlyAspLysSerValAlaMetValGlnIleValSerThrSerMetGluAsnMetTyrLeuT

     1610      1620      1630      1640      1650      1660      1670      1680      1690      1700
ATTTAACGCATGAATCAAAAGACGCTATTGCTAAAAATAAGCATAAATATTCTAAAGCTGATATTAGGTTGTTGAATAATTTTGATATTGATCGTTATGT
yrLeuThrHisGluSerLysAspAlaIleAlaLysAsnLysHisLysTyrSerLysAlaAspIleArgLeuLeuAsnAsnPheAspIleAspArgTyrVa

     1710      1720      1730      1740      1750      1760      1770      1780      1790      1800
AACGCTTGACGTCGAAGATAAAGACGATATGTTGAATGATGTTTGCGATTTAATTGATGATCATAATTTAGCAAATATGCGTGAATTAAGGCGCTTTGTT
lThrLeuAspValGluAspLysAspAspMetLeuAsnAspValCysAspLeuIleAspAspHisAsnLeuAlaAsnMetArgGluLeuArgArgPheVal

     1810      1820      1830      1840      1850    BanII/SacI/SstI     1880      1890      1900
AAATTACATGGGGTTGAATATGGCTTGCCTAGTATGAAAATTATAAATTCAGTGTTGAGAGCTCATACTGGACTGATAAGGTTATATTTCGATGCTGTTT
LysLeuHisGlyValGluTyrGlyLeuProSerMetLysIleIleAsnSerValLeuArgAlaHisThrGlyLeuIleArgLeuTyrPheAspAlaValT

     1910      1920      1930      1940      1950      1960      1970      1980      1990      2000
ATCAAGAACGTCGATATGGTCGGGCAGATATTGATAAAGATACAGGCGAAGTATTGAATAATAAGTAAGGGTTTAATTTTATTTTAGGGGGAAGCAATTA
yrGlnGluArgArgTyrGlyArgAlaAspIleAspLysAspThrGlyGluValLeuAsnAsnLys                    Start ORF3  M

     2010      2020      2030      2040      2050      2060      2070      2080      2090      2100
TGAAATTTGAAGAAACATTATTACCAGAAAAATCAGACGTAATGACATTACAAAATATGATCAGAAAGTATAATAAGCAAAATTTTGAAACTGCAAATCA
etLysPheGluGluThrLeuLeuProGluLysSerAspValMetThrLeuGlnAsnMetIleArgLysTyrAsnLysGlnAsnPheGluThrAlaAsnGl

     2110      2120      2130      2140      2150      2160      2170      2180      2190      2200
AACAGATTTTGCTATTTACATAAAAGATGATAGTGAGAATGTTATGGGGGGGATTTCAGGAAAAATATTTGGTAATTGGATGGATATAGACTATTTAGTT
nThrAspPheAlaIleTyrIleLysAspAspSerGluAsnValMetGlyGlyIleSerGlyLysIlePheGlyAsnTrpMetAspIleAspTyrLeuVal

     2210      2220      2230      2240      2250      2260      2270      2280      2290      2300
ATTCATGAGAGTTTGCGCAAAAATGGGTTAGGTCGGGATTTGTTAAAAAAAGCCGAAGATTTAGCTATACGGAGTAATTGTAAAAATATATTTTTATATA
IleHisGluSerLeuArgLysAsnGlyLeuGlyArgAspLeuLeuLysLysAlaGluAspLeuAlaIleArgSerAsnCysLysAsnIlePheLeuTyrT

     2310      2320      2330      2340      2350    SnaBI       2370      2380      2390      2400
CATTTGATTTTCAAGGTAAAGATTTTTATCCCAAATTTGGATTTAAAGAAGTTTACGTAAAAAGACAGTATCCGTTAACTGGAACAGAACATTTTTTTGT
hrPheAspPheGlnGlyLysAspPheTyrProLysPheGlyPheLysGluValTyrValLysArgGlnTyrProLeuThrGlyThrGluHisPhePheVa

<-- sequence in pJK352
                  ClaI 2430      2440      2450      2460      2470      2480
TAAGAAATTATCAAATGAAATCGATAAAGAAACTGAATGATGTGATGAAAGGACGGATAACTAGATCCGTCCTTTTTTGTTGTCGATTA
lLysLysLeuSerAsnGluIleAspLysGluThrGlu          ----------      ----------
                                                terminator
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FIG. 1. Nucleotide sequence of plasmid pLC2. The deduced amino acid sequences for the three open reading frames are given below the noncoding strand. Numbering starts with +1 at the first nucleotide of the *Sal*I site, used for cloning of the complete plasmid in vector pJK80. The nucleotides subcloned for construction of the vectors pJK352, pJK353, pJK354, pJK355, and pJK356 are marked. A possible plus *ori* is indicated. The inverted repeat (IR) between bp 574 and 640, proposed as minus *ori*, is underlined, with its highly conserved stem structure double underlined. The potential promoter sites upstream ORF1 and ORF2 as well as the positions of some pertinent restriction sites are shown. A stem–loop with a free energy value dG of −15.2 kcal/mol, followed by a stretch of T residues, which could be located downstream of ORF3 (bp 2448–2475) might function as a translational terminator, as predicted by the computer algorithm of Brendel and Trifonov (1984). Sequence data have been deposited with the EMBL/GenBank Data Libraries and DDBJ databases under Accession No. Z14234.

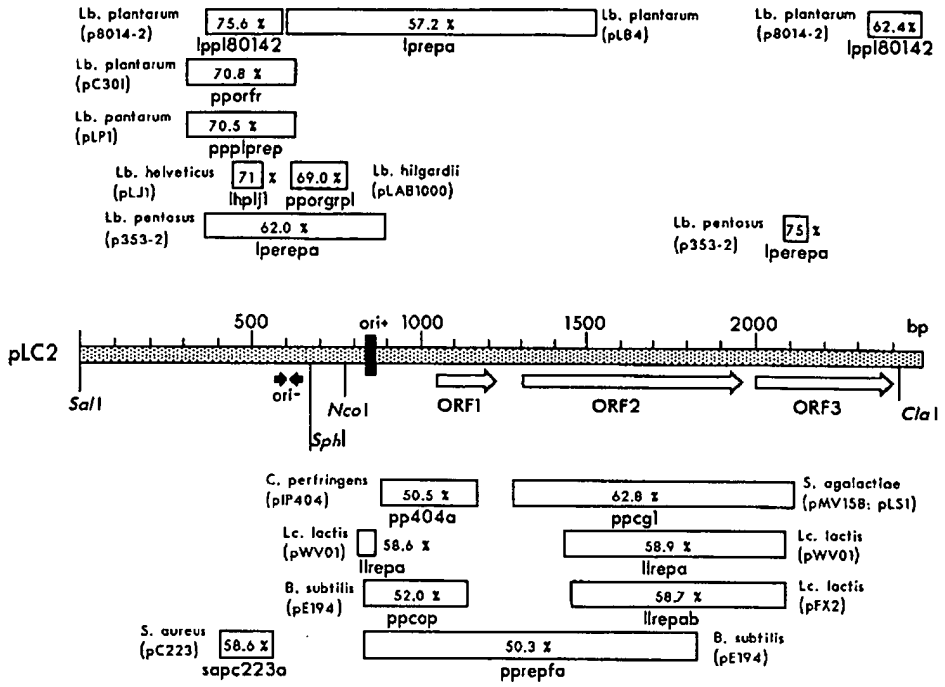

FIG. 2. Schematic alignment of pLC2 nucleotide sequence with homologous regions of plasmids from gram-positive bacteria. Sequence homology resulted from searching pLC2 against the complete EMBL database with the programs FASTA (Pearson and Lipman, 1988) and BLASTN (Altschul *et al.*, 1990). The stretches of homology, drawn to scale, are given as boxes with the EMBL entry name below. The bacterial host names of the different plasmids (names in brackets) are placed beside, and the degree of sequence identity resulting from FASTA analysis inside, the boxes. The complete plasmid pLC2 was searched against the database, thus resulting in maximum stretches of high homology. If the searched sequence is limited (e.g., isolated ORFs), different homology values are obtained. pLC2 with some pertinent features is given in the center of the drawing.

*Dra*III fragment, thus making the plasmid nonfunctional in *L. casei* LK1. The proposed *ori*+ (position 813–869), which is the palindromic sequence with the highest free energy in pLC2, possesses an internal stem–loop structure (Fig. 3B), which could be the signal for plasmid replication as it has been shown for plasmid pLS1 (De la Campa *et al.*, 1990).

The pLC2 minus origin. Other secondary replication functions in ssDNA plasmids are the minus origins (MO) of replication, palindromic sequences functioning as initiation sites for lagging strand synthesis. They are known to be located within regions of dyed symmetry (Bron *et al.*, 1988; Novick, 1989). This site is normally located outside the minimal replication region, and its deletion may influence stability and copy number (Gruss and Ehrlich, 1989). Different sequences representing the lagging-strand initiation site have already been characterized: palA (Gruss *et al.*, 1987), BA3 (Bron *et al.*, 1988), BAA1 (Devine *et al.*, 1989), and Leer *et al.* recently described a new type not belonging to these three groups, which is highly conserved in plasmids like p8014-2, pC30il, pLB4, and pLJ1 (Leer *et al.*, 1992).

Since typical minus origins as palindromic sequences of up to 200–300 base pairs in length have the potential to form secondary hairpin structures, the plC2 sequence was searched for such regions. The proposed *ori* plus site with a ΔG of -20.8 kcal/mol was found, but using the available computer software, only weak (< -15 kcal/mol), short (approximately 10 bp), and imperfect structures

A

Plasmid				Host of origin	Referrence
pLS1	427	AAAAtATg GGGGGGc	TACTACGA cCCCCCCtaT AGTG CC GAGTGCCAA	S. agalactiae	De la Campa et al., 1990
pLB4	2004	AAAAcAT GGGGG	TACTACGAcacCCCCCCAT GTGtCC attgtCCAt	Lb. plantarum	Bates et al., 1989
pE194	870	AAAAcATa GGGGGG	TACTACGAcctCCCCCCtag GTGtCCattGT CCAt	S. aureus	Grus and Ehrlich, 1989
pAD8201	57	tcAcgAccaGGGGGc	TACTACGATag CCCCCAaT gAGTGatt tGTGaCAt	M. mycoides	Grus and Ehrlich, 1989
pSH71		AAAAAAT GGGGG aaaggaagcgaattttgcttccg	TACTACGA CCCCCATT AAGTG CC GAGTGCCAA	Lc. lactis	Grus and Ehrlich, 1989
pFX2	1087	AAAAAAT GGGGGG aaaggaagcgaattttgcttccg	TACTACGA CCCCCCATT AAGTG CC GAGTGCCAA	Lc. lactis ssp. lactis	Xu et al., 1991
pWV01	329	AAAAAAT GGGGGG aaaggaagcgaattttgcttccg	TACTACGA CCCCCCATT AAGTG CC GAGTGCCAA	Lc. lactis ssp. cremoris	Leenhouts et al., 1991
		-------> <--------			
pLC2	813	AAATcaGGGGGGcg agggagcgaattttgcgcccg	TACTACGA CCCCCCTTTTAAGTG CC GAGTGCCAAAT	Lb. curvatus	this work

B

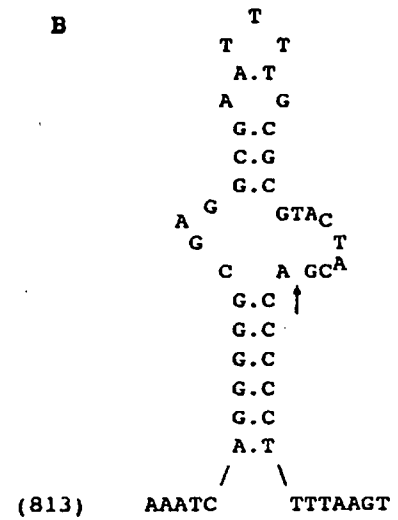

FIG. 3. Comparison of plasmid plus *ori* sites. (A) A conserved inverted sequence of 23 bp in pFX2 and pSH71 is marked by facing arrows (Xu *et al.*, 1991). A similar sequence, but having less potential to form a stem-loop structure, can be located in pLC2 at the same position. (B) A possible loop structure in the putative plus *ori* locus of pLC2 with a free energy of −20.8 kcal/mol. The putative nicking site, as found in pLS1, is indicated by an arrow.

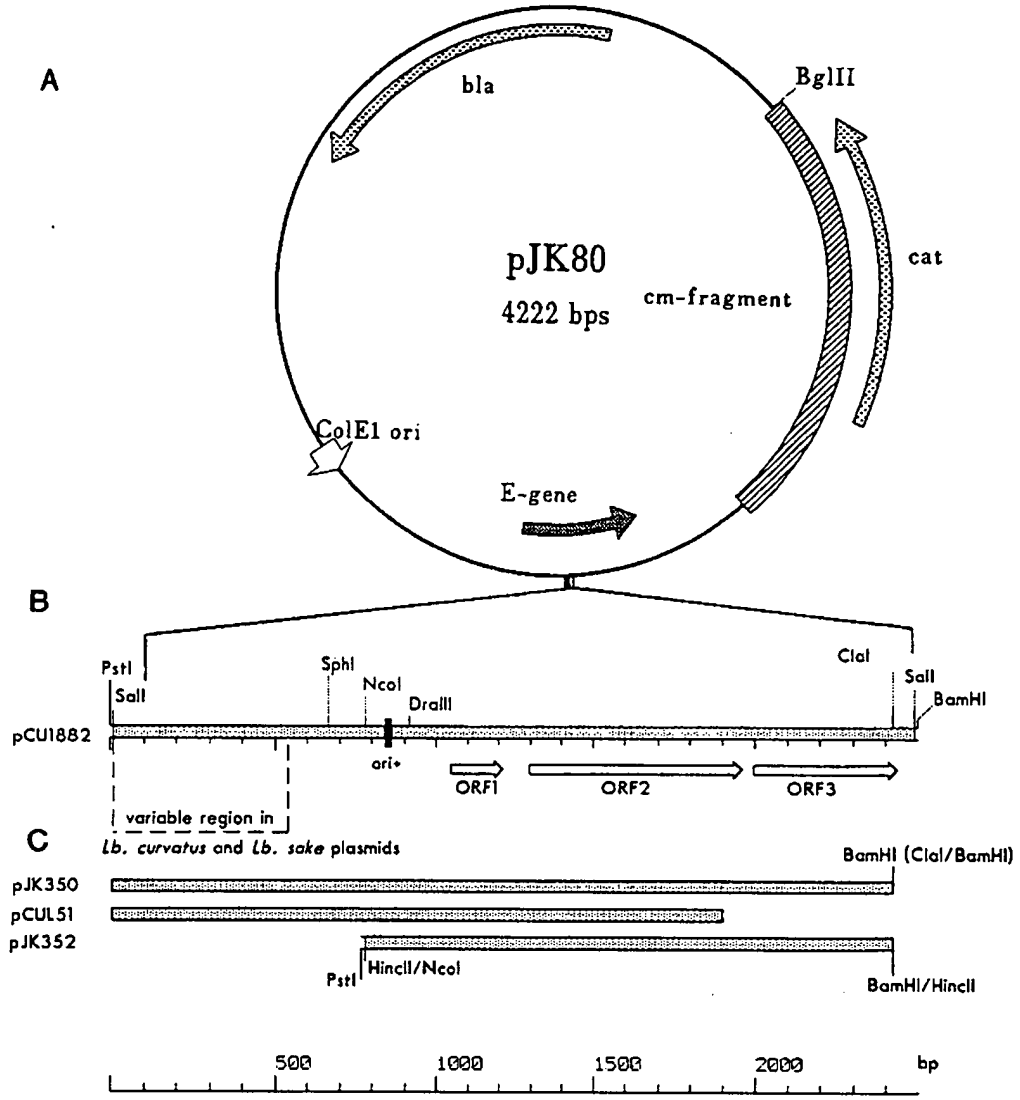

FIG. 4. Determination of the minimal replicon of pLC2. (A) Cloning of plC2 as *Sal*I fragment in pJK80 (for details see bacterial strains and plasmids) resulted in pCU1882. (B) The variable region, described by Vogel *et al.* (1991) in the noncoding part of the plasmid, is indicated. (C) All the plasmids presented replicate in lactobacilli. pJK350 arose from pCU1882 by deletion of a 75-bp *Cla*I/*Bam*HI fragment. Exonuclease III treatment of pCU1882, linearized with *Cla*I, resulted in pCUL51, with a deletion reaching into ORF2 (position 1909) as examined by sequence analysis. Plasmid pJK352 was obtained after subcloning the *Nco*I/*Bam*HI fragment from pJK350, treated with Klenow enzyme, into the *Hin*cII site of pJK80.

could be additionally localized, which did not resemble a typical minus origin. But if the results from homology comparison with other plasmids were examined carefully, a region upstream the pLC2 *ori+* site, which is not coding for proteins, shows strong homology to *L. plantarum* and *L. pentosus* and *L. helveticus* plasmids (Fig. 2). Between bp 305 and 640, pLC2 has more than 70% identity to the *L. plantarum* plasmids pLP1 (Bouia *et al.*, 1989), pC30il (Skaugen, 1989), p8014-2 (Leer *et al.*, 1992), and pLB4 (Bates and Gil-

bert, 1989); to the *L. pentosus* plasmid p353-2 (Leer *et al.*, 1992); and to the plasmid pLJ1 from *L. helveticus*. As reported by Leer *et al.* (1992), these plasmids contain a new type of minus *ori*, which is just in the region showing homology to pLC2. Based on these data an imperfect stem–loop structure with a free energy of −20 kcal/mol could be calculated. This free energy and the size of the structure is in the range of the values of the inverted repeats (IR) which were found for plasmids having the new type of minus origin. The stem of the IR is almost perfectly conserved (double underlined in Fig. 1), whereas the loop with weaker base pairing is not. Upstream of this IR there is a longer stretch of homology (position 305–640 of pLC2) to other plasmids. Leer *et al.* (1992) have found that the difference in size between the closely related *L. plantarum* plasmid (p8014-2) and the *L. pentosus* plasmid p353-2 is an effect of variability in this region upstream of *ori*+. This notice agrees well with the observation made by Vogel *et al.* (1992) after comparative restriction analysis of different *L. sake* and *L. curvatus* plasmids. They noticed a highly conserved and a variable region for all plasmids (including pLC2) observed and the sequencing data obtained from pLC2 now allow to appoint this region (Fig. 4). It is identical to the DNA stretch with the IR capable to be the MO and the region upstream which might have regulatory functions or incompatibility properties but which is not coding for proteins.

Protein coding regions. Downstream of the *ori* site, three open reading frames, ORF1 (position 1049–1216), ORF2 (position 1303–1965), and ORF3 (position 2000–2437), were located, with 56, 221, and 146 codons, respectively (Fig. 1). The method developed by Kolaskar and Reddy (1985) allowed the prediction for all three reading frames to have high coding probabilities. Possible coding regions could not be located on the lower strand of the plasmid.

ORF1 is proceeded upstream by a 69-bp sequence of 84.1% A+T (958–1026), which could serve as −35 and −10 sequence of a promoter, followed by a potential ribosome-binding site (RBS) aAGGGA at position 1036. ORF1 shows homology to the cop protein of the *S. aureus* plasmid pE194 (Byeon and Weisblum, 1990).

Twelve base pairs upstream from the ATG site of ORF2, a potential RBS (TAAGG), preceded by a promoter −10 sequence TATAAT, can be localized. Whether expression signals of lactobacilli have the same conserved features as other gram-positive bacteria and determination of the kinds of changes in primary sequences that affect the efficiency of gene expression require further investigation.

Searching the nucleotide or deduced amino acid sequence of ORF2 against a data base resulted in homologies to the replication proteins of the pE194 group plasmids, which agrees with the classification of the pLC2 *ori*+ sequence being the corresponding target site of the rep protein. The complete gene product of ORF2 has 59.9% homology to the replication protein of pMV158 or pLS1 and 48.1% identity to repA of plasmid pWV01.

Interestingly the plasmids which have a similar MO as pLC2 do not belong to the pE194 class but have *ori*+ and corresponding *rep* genes as the pC194-type RCR plasmids. This favors the theory of Jossen *et al.* (1990) that plasmids might consist of functional cassettes, which can be exchanged by horizontal transfer through the different genera.

In vitro transcription/translation assay. Evidence that the predicted protein coding regions ORF1, ORF2, and ORF3 are encoding was obtained by coupled *in vitro* transcription and translation of plasmid pLC2. The plasmid was shown to direct the synthesis of proteins with molecular weights of approximately 27, 17, and 9.5 kDa (Fig. 5). These products agree quite well with the predicted sizes of 25.688 (ORF2), 17.210 (ORF3), and 6.633 (ORF1). Nevertheless it should be kept in mind that genes of *L. curvatus* are transcribed and translated by an *E. coli*-derived system and heterologous genes are not expected to be always well expressed.

Replication of pLC2 via a RCR mecha-

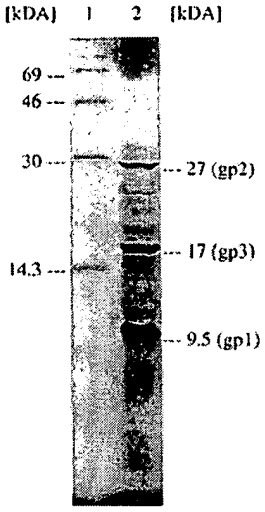

FIG. 5. Autoradiogramm of the [^{35}S]methionine-labeled proteins produced by *in vitro* transcription/translation and separation by SDS-PAGE (15% acrylamide). The gene products (gp) proposed to be encoded by the three open reading frames are labeled gp1, gp2, and gp3, respectively. Molecular masses of [^{14}C]-methylated protein markers are indicated.

nism. The sequence homology between the pLC2 plus origin to the pE194-type plasmids, as well as the imperfect repeat (bp 594–640) to a minus origin and the similarity of the protein coding regions to other gram-positive plasmids, suggested that pLC2 is like all these other related plasmids replicating by a RCR mechanism. To prove the evidence, whole cell lysates of LTH683 were electrophoretically separated and intermediate DNA was detected after hybridization with a digoxgenin-labeled pLC2 probe. As demonstrated in Fig. 6 only a faint ssDNA band could be detected. The proof that the fast running DNA was single stranded could be performed by digestion with nuclease S1. It is generally believed that conversion to dsDNA is initiated by host RNA polymerase (Gruss and Ehrlich, 1989) and that the leading strand is primed by antisense RNA. Therefore growing cells were inhibited with rifampicin, an inhibitor of RNA polymerase and after incubation with rifampicin, ssDNA significantly accumulated. The very low amount of ssDNA in untreated cells suggested that ssDNA is very efficiently converted into dsDNA. The occurrence of a ssDNA intermediate reinforces the opinion that pLC2 replicates via a RCR mechanism.

The essential region of pLC2 necessary for replication. Deletion derivatives of pCU1882 were constructed and tested for autonomous replication in lactobacilli. The essential region for plasmid pLC2 (as summarized in Fig. 4) could be located within basepairs 780 (*Nco*I site in pJK352) and 1909, being the termination site of exonuclease III deletion in pCUL51. Therefore the genes coded by ORF1 and ORF2 were sufficient for autonomous replication. Additionally the IR (bp 574–640 in pLC2) proposed for being the minus origin is non essential for replication. The comparative studies on small cryptic plasmids from *L. curvatus* and *L. sake* by Vogel *et al.* (1991) revealed a variable region among the different plasmids and this variable stretch in pLC2 corresponds to the deletable *Sal*I/*Sph*I fragment (bp 1–664) removed in pJK352 and all its derivatives (Fig. 4). This may suggest an increased instability accompanied with accumulation of ssDNA but this has to be further investigated in detail.

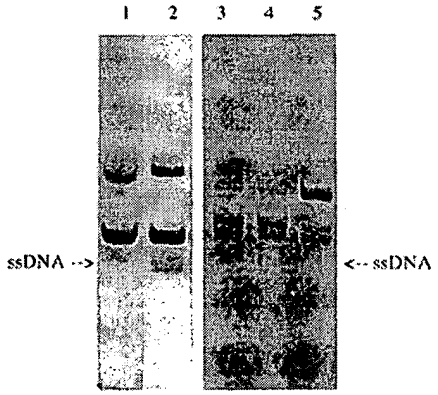

FIG. 6. Whole cell lysates of *Lb. curvatus* LTH683 hybridized with digoxigenin-labeled pLC2 probe. A faint ssDNA band detectable in untreated cells (lane 1) accumulated after inhibitin with rifampicin (lane 2). Prior treatment with S1 nuclease, DNA was precipitated with ethanol, washed, and resuspended in the original volume. 0 units S1 (lane 3), 0,4 units (lane 4), 4 units (lane 5).

Construction of Lactobacillus Cloning Vectors

The sequence of plasmid pCU1882, containing the whole cryptic plasmid pLC2 as *Sal*I fragment in the vector pJK80, could be deduced from the established pLC2 nucleotide sequence and the vector sequence. The deletion derivative pJK352 was chosen for the construction of vectors with multiple cloning sites. Since pJK352 contains two replicons, the pLC2 origin and ColE1, the latter should be deleted after insertion of a polylinker fragment. In advance it has been shown that the deletion of the ColE1 replicon in pJK352d (Fig. 7A) had no effect on the ability of replication in lactobacilli.

A multiple cloning site was designed for pJK352 by choosing rare cutting restriction enzymes sites not present in the plasmid. Two complementary single-stranded 73 mer were synthesized (Fig. 7B) and annealed to double-stranded DNA. *Pst*I ends were generated with the appropriate restriction enzyme and ligated into the phosphatase-treated *Pst*I site of pJK352. Transformation of the ligase mix and isolation of plasmids was carried out in LK1. The two possible orientations of the polylinker fragment could be determined by examining the DNA fragments produced by restriction with *Ava*I/*Bst*XI and resulted in plasmids pJK353 and pJK354. The ColE1 origins of these plasmids were removed (Fig. 7A), resulting in the vectors pJK355 and pJK356.

Plasmid stability. For application in industrial fermentation processes, it is in many cases desirable that the vector be stably maintained in the host cell, even in nonselective medium. Transformants of LK1 and LTH1432 with plasmids pCU1882, pJK355, and pJK356 were incubated without selective pressure. No significant loss of plasmid could be detected after incubation for 56 generations (>90% cells contained plasmid), but after an additional transfer (equivalent to 70 generations) only 75% of plasmid remained, and after another transfer (84 generations), the plasmids pJK355 and pJK356 were lost to 40%. The content of pCU1882 remained unchanged. This segregational instability, probably a result from deleting the *ori*−, was not accompanied by structural instability. The minimization of the replicon lead to a slight loss of stability, but because in many food fermentation processes the inoculum with starter cultures is very high and bacteria undergo only few generations (Vogel *et al.*, 1992), the cloning vectors constructed may be useful in lactobacilli.

Host range. After transformation of plasmids pJK352d and pJK355 or pJK356 into the heterologous gram-positive strains *B. subtilis* 6GM and *L. lactis* ssp. *lactis* MG1363, chloramphenicol-resistant colonies could be propagated in liquid culture and plasmid DNA isolated was unchanged in its electrophoretic pattern. Transformation of *E. coli* cells resulted in small chloramphenicol-resistant colonies, but no growth was obtained in liquid medium. This indicated an unsuccessful replication of the lactobacillal replicon in *E. coli*. Selection with chloramphenicol was possible as demonstrated with pCU1882 and other derivatives having both origins pLC2 and ColE1. But all these plasmids had a tendency to delete DNA fragments after several passages in *E. coli*, whereas in *Lactobacillus* this phenomena was not observed. This observation suggested that ColE1 sequences are not expressed in lactobacilli and do not impair the segregational stability, whereas in *E. coli* the lactobacillal genes or structures are interfering with the replication mechanism of the *E. coli* vector.

Possible applications of the vectors. If a cloned potential food grade marker gene, with selectable phenotype, or having the capacity to deliver a benefit in growth, is inserted into the cloning sites of pJK355 or pJK356, the unwanted chloramphenicol resistance gene might easily be removed due to the presence of many single rare cutting restriction enzyme sites. The proposed strategy, demonstrated in Fig. 8, would result in a plasmid with the quality of being useful in food industry.

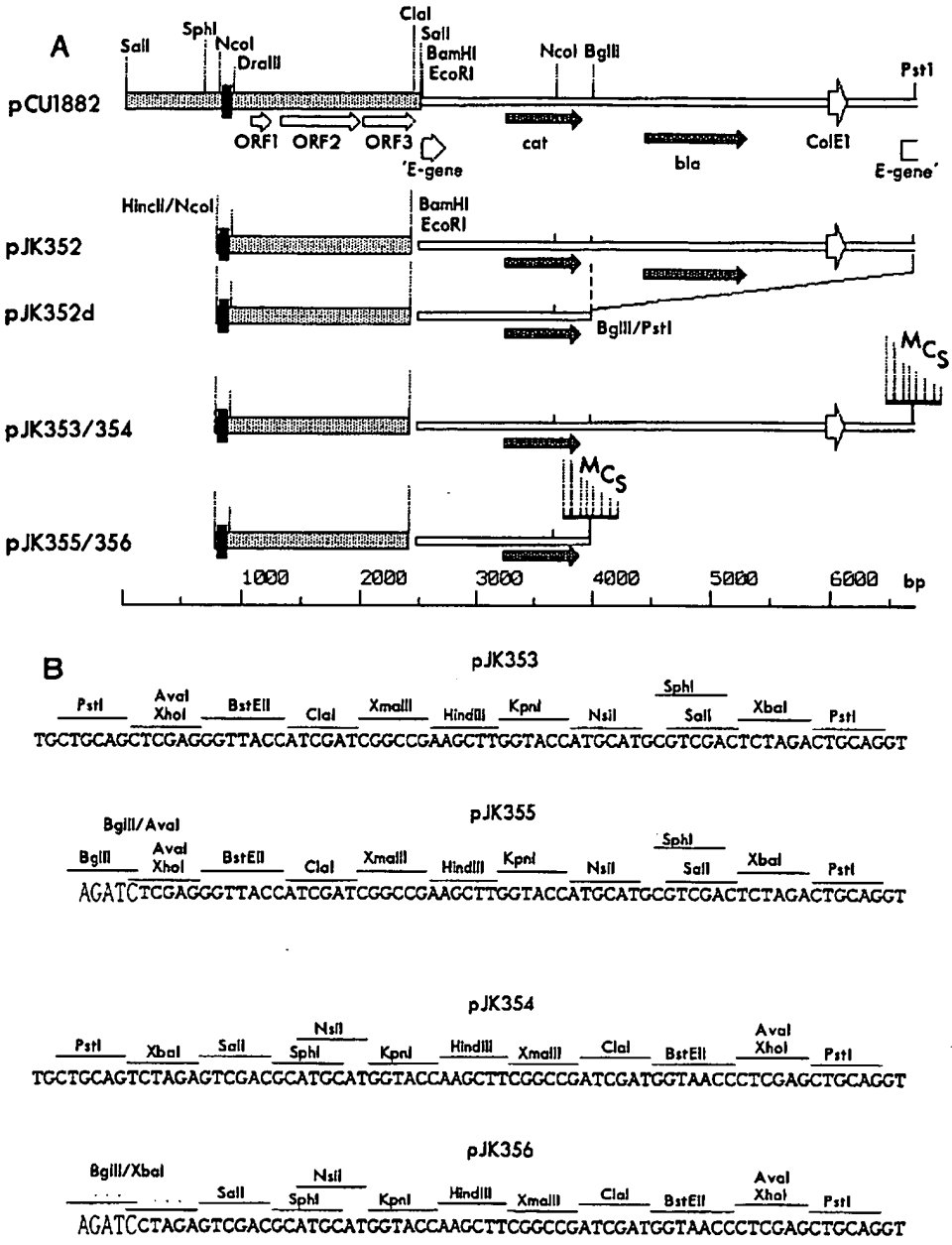

FIG. 7. (A) Construction of cloning vectors pJK355 and pJK356. pCU1882 contains the complete plasmid pLC2 (shaded box) in pJK80 (open box). The antibiotic resistance genes (cat, chloramphenicol transcetylase; bla, β lactamase) are indicated by dotted arrows. Plasmid pJK352 deleted for the "variable DNA region" (Fig. 4) was commencing product for vector construction. pJK352d served as a control, confirming that deletion of ColE1 had no effect on replicational properties. The synthesized polylinker fragment was integrated into the *Pst*I site of pJK352 and the two possible orientations resulted in pJK353 and pJK354. For the deletion of the ColE1 origins pJK353 was restricted with *Bgl*II and *Ava*I, ends were filled in with Klenow enzyme and religated. The resulting plasmid pJK355 retained the *Bgl*II and *Ava*I sites. pJK354 was cut with *Bgl*II and *Xba*I, and blunt ends were created with Klenow enzyme and religated. The construct pJK356 had lost the restriction sites for *Bgl*II and *Xba*I. (B) Nucleotide sequence of the multiple cloning sites in plasmids pJK353, pJK354, pJK355, and pJK356, with orientations corresponding to Fig. 7A.

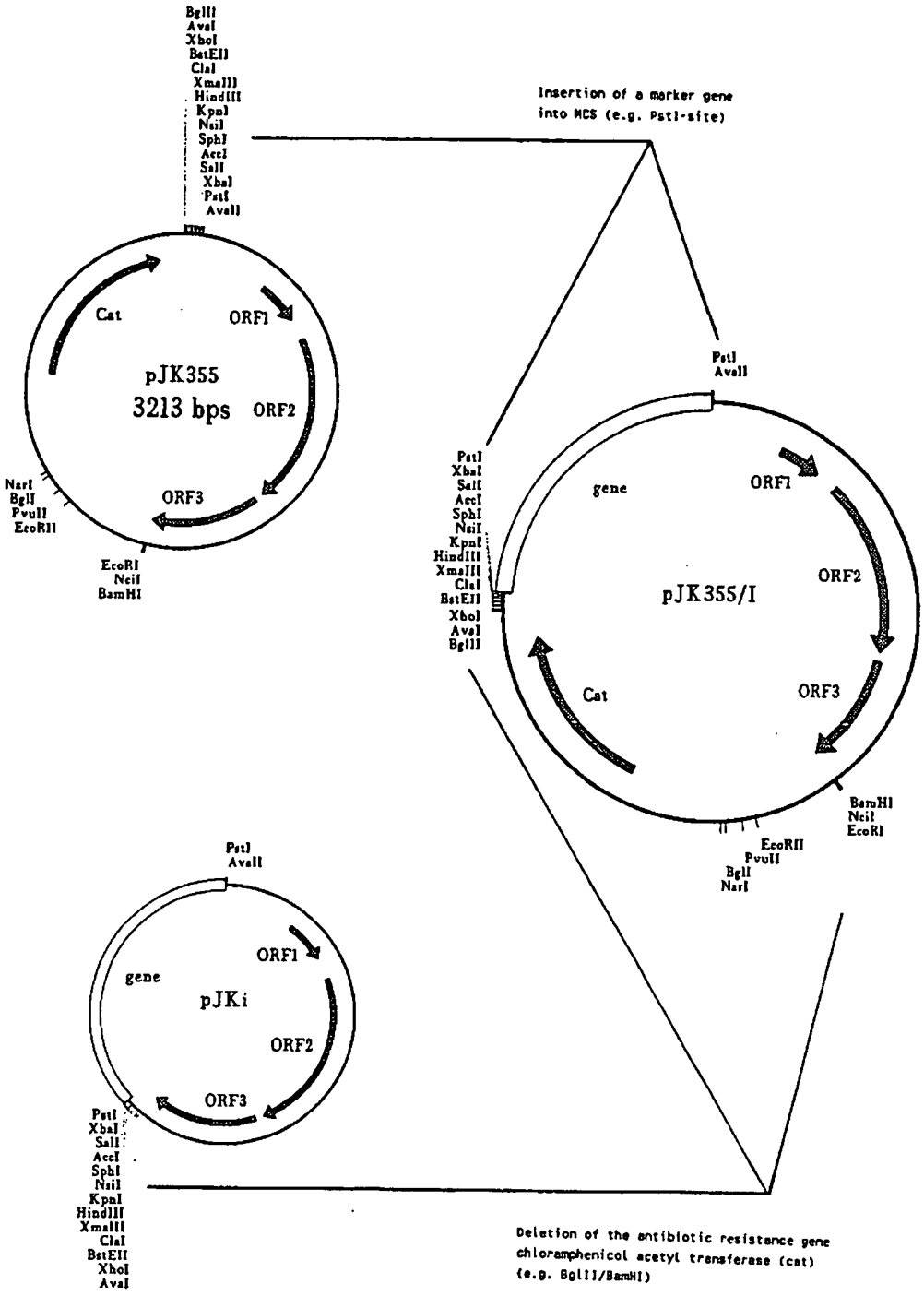

FIG. 8. Replacement of the chloramphenicol resistance gene in pJK355 with a selectable food grade marker gene. All restriction sites listed are unique. The MCS of either pJK355 or pJK356 can be used to insert a gene in that way that the *cat* gene can be eliminated by restricting the plasmid with a pair of single cutting enzymes followed by recircularization.

To prove the usefulness of the vector for cloning and homologous expression in a *Lactobacillus* starter culture, we inserted the recently cloned *pepN* gene, coding for the aminopeptidase N (EC 3.4.11.2) from *L. delbrückii* ssp. *lactis* WS87, as 5.2-kb *Sph*I-restriction fragment, into pJK355 and pJK356. In *L. casei* LK1 harboring the resulting hybrid plasmids, peptidase N activity was increased by 15- to 18-fold (data not shown). More details about cloning of the *pepN* gene will be published elsewhere and investigations will be made if the outlined strategy for eliminating the resistance gene can be applied in this special case.

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